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Development and Validation of Gc-Hs Method for the Determination of Residual Solvents in Fluconazole

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ABSTRACT

A simple GC-HS method for the determination of residual solvents in fluconazole drug using Nitrogen as the carrier gas at 2.8 mL/minwith ZB-624(30 meters X 0.53 mm ID) as column using FID as detector is developed. The developed method is validated and the parameters are to be found within the limits of USP. The retention time for residual solvents individually and in standard solution is determined. The %RSD for six injections is found to be NMT 15%. The correlation coefficient R2 is found to be ≥ 0.999. The limit of detection and limit of quantification are found to be specific. Method precision is found to be within the acceptance limit. The sample is tested for the presence of residual solvents mainly Methanol, Acetone, Dichloromethane, Ethyl acetate, Toluene, and Isopropyl alcohol, which are found to be within ICH limits.

KEYWORDS :

INTRODUCTION

Fluconazole (brand name **Diflucan)** is an antifungal agent.Fluconazole is chemically 2-(2, 4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)-2-propanol, a synthetic triazole derivative antifungal agent(Figure 1). It is known to be used for superficial and systemic fungal infections[1]. It interacts with 14-α demethylase, which is needed to convert lanosterol to ergosterol. Ergosterol is an essential component of fungal cell membrane. By inhibiting the synthesis of this component, results in leaky fungal cell. This results in leakage of cellular contents.

Residual solvents are the organic volatile chemicals that are used or produced in the manufacture of drug substance, excipients or in the preparation of drug products. These solvents are not completely removed by practical manufacturing techniques. Since there is no therapeutic benefit from residual solvents should be removed. Gas chromatographic method is most sensitive method for analysis of residual solvents[2].

In literature, several analytical methods are found for quantitation of fluconazole such as spectroscopic determination of fluconazole in pharmaceutical dosage forms and in human plasma by HPLC, UV and GC available. But there is no GC method for the estimation of residual solvents in the flucanozole. So for the first time, an attempt has been made to develop and validate a simple, rapid and highly selective HSGC method[3,4] for the quantification of residual solvents in Fluconazole. The method was validated as per ICH guidelines [5,6]. The method was shown to be specific and was applied successfully to monitor and control these solvents on a manufacturing level[7,8]. The method was found to be applicable for the routine analysis of residual solvents in Fluconazole and in pharmaceutical formulation [9].

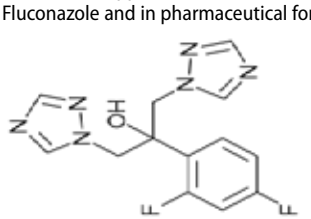

Figure 1: Chemical structure of Fluconazole

EXPERIMENTAL

Instrument:

Headspace Gas Chromatography: The analysis is performed on Shimadzu Gas Chromatography, model no Shimadzu-GC-2010 plus and Teledyne –Teckmar head-space, AOC 5000 auto sampler and a flame-ionization detector. The optimized chromatographic conditions, temperature programming and head space programming are presented in Table 1,2,3.

Table 1: Optimized chromatographic conditions

Column	ZB-624
Dimension	30 meters x 0.53 mm ID (5µm)
Detector	FID
Detector Temperature	280^{0}C
Injector Temperature	140^{0}C
Injector volume	1.0 mL vapor
Runtime	28.33 minutes
Carrier Gas	3.5 mL/min. (Nitrogen)
Column flow	2.8 mL/min
Zero air flow	400 mL/min
Hydrogen flow	40 mL/min
Split ratio	1:10

Table 2: Temperature program for GC Column:

Rate	Temperature	Time (in min)
	35°C	5
10°C	100°C	3
15°C	150°C	3
20°C	240°C	3

Table 3: Head space programming

Equilibrium temperature	90°C
Transfer-line temperature	110°C
Oven temperature	100°C
Time	22min.

METHOD DEVELOPMENT

Drug and chemicals:

Fluconazole drug was obtained from Hetero drugs Pvt. Ltd. and marketed product was procured from local pharmacy. Residual solvents like methanol, acetone, dichloromethane, ethyl acetate, toluene, isopropyl alcohol and dimethyl formamide used were of HPLC grade and obtained from Merck Ltd.

Blank preparation:

Accurately 5.0 ml of dimethyl formamide (diluent) is pipetted out into a 20 ml headspace vial and closed with cap. This solution is termed as blank. 1 ml vapor of blank solution is injected, using auto injector.

Mixed Standard Solution preparation:

Accurately 3.8 µl of methanol, 6.3µl of acetone, 0.5µl of dichloromethane, 5.6µl of ethyl acetate, 1.0µlof toluene, and 6.4µl ofisopropyl alcohol are transferred into a 50 ml volumetric flask containing 20ml of diluent. The solution is mixed well and the volume is made up to the mark with dimethyl formamide (diluent). From this 5.0 ml of solution is pipetted into a 20 ml head space vial.

Procedure for calibration curve:

A method was developed by performing several trials and finally parameters were selected based on the acceptance limits of ICH. Each 1ml of blank, mixed standard and sample were injected into Head space and their chromatograms were recorded.

METHOD VALIDATION

The parameters like specificity, linearity, precision, robustness, ruggedness, system suitability, accuracy, batch analysis, LOD and LOQ are performed as those are mentioned in the International conference on harmonization (ICH) guidelines [4].

System suitability

System suitability is performed to ensure that the complete testing system is suitable for intended application.System suitability study of the method is carried out by injecting a blank i.e.; Diluent (Dimethyl Formamide) and six replicate analysis of mixed standard solution. Various chromatographic parameters such as retention time, peak area, tailing factor, theoretical plates of the column and resolution between the peaks are determined.

Specificity

It is performed to know the retention time for the residual solvents individually and in spiked sample solution.The individual and mixed standard solution in DMF is prepared at the working concentration level for each Methanol, Acetone, Dichloromethane, Isopropyl alcohol, Ethyl Acetate and Toluene. The blank preparation and individual solution are transferred in Headspace vials. Each vial is chromatographed using the headspace conditions. Peak response in the blank preparation was also recorded (figure2). The retention time for Methanol, Acetone, Dichloromethane, Isopropyl alcohol, Ethyl Acetate, Toluene, and DMF peaks are recorded (Figure3). Spiked sample solution is also recorded for retention times of residual solvents(figure 4).

Linearity

It is done to know the test results which are directly proportional to the concentration of analyte in the sample. Linearity study of the method is carried out by injecting a blank i.e.; Diluent (Dimethyl Formamide), each of three 50%, 75%, 100%, 125% and 150% solutions of mixed standard into the head space and calibration curves were plotted by taking response on the Y-axis and concentration on the X-axis(Figure 5).

Precision

It is validated to know the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Method Precision is carried out by injecting a blank i.e.; Diluent (Dimethyl Formamide), 6 replicates of mixed standard solution and 6 replicates of pure drug sample solution, each are of 100% in concentration into the head space.

Robustness

Robustness is tested by introducing small variations in method parameters. This study is performed by making small but deliberate variations in the method parameters. The effects of variation included are analysis with original temperature (35°C) and the column flow (2.8ml/min), ±5°C (i.e.40°Cand 30°C) and ±0.2ml/min (3ml/min and 2.6 ml/min) change in the column oven temperature and column flow were maintained. The variation in Headspace parameters are also performed, Headspace vial equilibration time is changed from 30min±5min, variation of the vial equilibration temperature of head space study is performed by changing from 90°C±5°C respectively and chromatograms were recorded. A blank i.e.; Diluent (DMF), 6 replicates of mixed standard and a pure drug sample solution were also introduced into the head space.

LOD and LOQ

These are determined by signal-to-noise ratio (S/N) method. A blank i.e.; DMF and six replicates of standard solution containing each solvent around its QL concentration were prepared and injected in chromatograph. Detection limit and quantitation limit values were determined.

Ruggedness

Ruggedness study of the method is carried out by injecting a blank i.e.; DMF, 6 replicates of mixed standard and a pure drug sample solution by two different analysts on two different days.

Accuracy

Accuracy is the amount of drug recoveredfrom the spiked sample. It is assessed by 9 determinations over a minimum of 3 concentration levels covering the specifiedrange.

Batch Analysis

Finally the sample is checked for the presence of residual solvents. Batch Analysis was carried out by injecting a blank i.e.; DMF, 6 replicates of mixed standard solutions and 2 pure drugs sample solutions of a batch into the head space.

Preparation of marketed formulation:

A weighed quantity equivalent to 100mg of fluconazole marketed formulation was transferred into 20ml headspace vial and 5ml of dimethyl formamide(diluents) was added to the same vial fitted with septum and sealed.

RESULTS AND DISCUSSION

Method development

Column selection:

The primary aim of the column selection was to resolve the solvents (Methanol, Acetone, Dichloromethane, Isopropyl alcohol, Ethyl Acetate and Toluene) which are utilized in the process of synthesis of Fluconazole or excipients. Several trials were done with different wall-coated capillary columns having different stationary phases and dimensions in order to separate and quantify solvents present in Fluconazole or excipients. For eg.,DB-624 column (30m length,0.52mm i.d with a stationary phase of 6% cyano propyl phenyl& 94% Dimethyl poly siloxane film of 3.5µ), ZB-624 column(30m length,0.52mm i.d with a stationary phase of 6% cyano propyl phenyl & 94% Dimethyl poly siloxane film of 5µ). Finally the ZB-624 column was found to be the best one for separation of all the 6 solvents in less time.

Thermal programming:

A linear thermal gradient was selected to provide elution of the solvent's peak during the chromatographic run for better resolution and quantification. Several trials were performed by changing linear ther-

mal gradient, among them an initial hold of 5min at 35°C and linear thermal gradient to 100°C at 10°C/min for 3min followed by 150°C at 15°C for 3min and 240°C at 20°C for 3min was found to elute better peaks showing the resolution more than 2.

Headspace method optimization:
The headspace method was finalized in such a way that 6 solvents present in the sample should vaporize for the detection. For this sample and standard vials were heated at 100°-90°-110°C for 30-25-35min with constant shaking. Among them a combination of sample vial heating at 90°C for 22mins shaking was found to suitable for getting better response.

Method validation

1. System suitability: System suitability parameters like asymmetry and resolution were calculated to evaluate the chromatographic parameters. The number of theoretical plates for the six replicate injections of mixed standard solution was found to be more than 3000, tailing factor was found to be less than 2 and the resolution between any two adjacent peaks were more than 2.0. The system suitability parameters were found to be in the acceptable range, which indicates suitability of system for the quantification of these 6 solvents by this method. The results obtained are presented in the Table.2.

2. Specificity: The blank chromatogram did not show any interference with the solvent peaks. Rt of the solvents peaks of the sample are compared with Rt of individual residual solvents and Rt values for Individual Methanol, Acetone, Dichloromethane, Isopropyl alcohol, Ethyl Acetate and Toluene were found to be 4.897min, 7.575min, 7.823mins, 8.436min and 11.472min and 16.447 and Rt values of spiked sample of Methanol, Acetone, Toluene were found to be 4.897min, 7.575min, 16.447mins. The results are shown in the Table 3.

3. Method Precision: Method precision was done by injecting one batch of sample at 100% concentration for six times. For each solvent, from chromatogram peak areas % Relative standard deviation was calculated. % Relative standard deviation for four solvents was found to be less than 15% hence the method is precise and given in the Table.4.

4. Linearity: Linearity is performed from 50-150% and graphs obtained from the linearity were observed to be linear and showing correlation coefficient R2≥0.998%. Calibration curves are plotted by taking response on the Y axis and concentration on the X axis (Linearity range, correlation coefficient and slope values are tabulated in Table 5.

5. Detection (DL) and Quantization (QL) Limit: Solution containing individual solvent was prepared around its QL concentration and injected in six replicates. The DL and QL for all solvents were determined by signal-to-noise ratio (S/N) method. From these limits, it was observed that the minimum concentration (ppm) is at 3:1 S/N (for DL) and the quantification concentration is at 10:1 S/N (for QL) and the DL values for Methanol, Acetone, Dichloromethane, Isopropyl alcohol, Ethyl Acetate and Toluene were found to be 0.001887, 0.000779, 0.001542 ,0.019349,0.000913 and 0.003050 respectively and the QL values were found to be 0.0062271, 0.0025707, 0.005886, 0.0638517, 0.0638517, 0.0030129 and 0.010065 respectively. The values are given in the Table.6 and 7.

6. Robustness: Robustness of the method was performed by making small variations in the optimized parameters. There were no marked changes in the %RSD of the areas of solvent peaks. From the results it was observed that the method remain unaffected even a slight changes in the optimised conditions and the values are presented in the Table 8.

7. Ruggedness: Analysis was performed by different analyst on different days by injecting six replicates of the mixed standard solution into the optimized chromatographic system. %RSD was calculated from the data obtained and it was found that the %RSD values was less than 15% for all the 6 solvents although the analysis was performed on different days by different analysts hence the method is said to be rugged. The results obtained are tabulated in the Table. 9.

8. Accuracy: Accuracy of the method was done by recovery experiments by spiking known amount of each solvent at quantization limit, 50%, 100% and 150% of 5000 ppm to the test solution. Each preparation was analyzed in triplicate and percent recovery was calculated. The recovery values were found to be between 90.29% and 99.66% and results obtained were within the limits and are summarized in the Table 10.

9. Batch Analysis: Batch analysis was performed by injecting test samples and a formulated product of a batch and whose results were found to be within the limits and the values for Methanol, Acetone and Toluene were found to be 39ppm, 8ppm and 33ppm. The assay results are presented in the Table.11.

CONCLUSION:
A single, simple and rapid GC-HS method is developed for determination of residual solvents in fluconazole with FID detector. The individual solvents were clearly separated on ZB-624 column with a flow rate of 2.8ml/min and mobile phase of nitrogen. Residual solvents are used in the manufacturing of drugs and excipients at various steps but these solvents are harmful to the health. As these solvents cannot be removed completely they should within the limits as per ICH guidelines.The method is validated for specificity, linearity, precision, batch analysis, system suitability, LOD and LOQ. All the validated parameters were found to be within the ICH limits. This method was successfully used to estimate the residual solvents present in the fluconazole pure drug and marketed formulation.

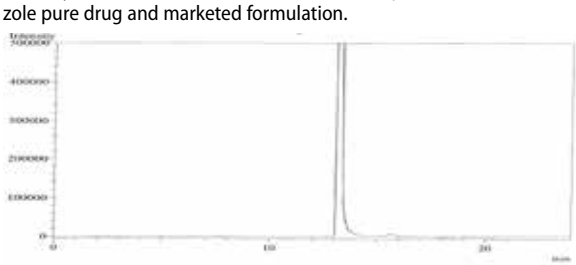

Fig 2: Chromatogram of blank

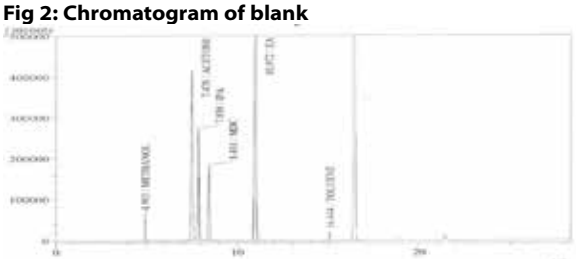

Fig 3: Chromatogram of standard

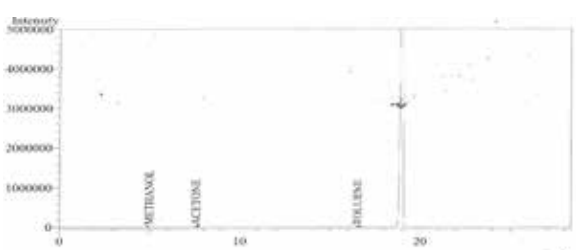

Fig 4: Chromatogram of sample

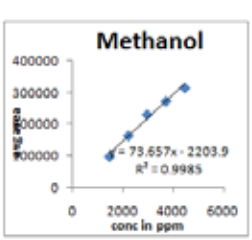

Fig 5a: Calibration plot of Methanol.

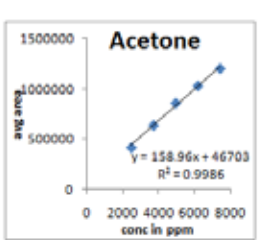

Fig 5b: Calibration plot of Acetone.

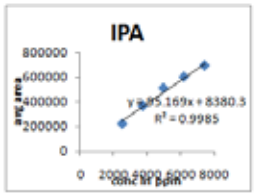

Fig 5c: Calibration plot of IPA.

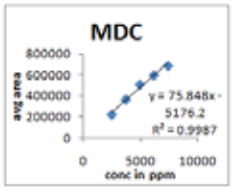

Fig5d: Calibration plot of MDC.

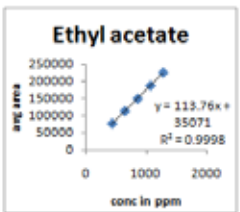

Fig 5e: Calibration plot of EA.

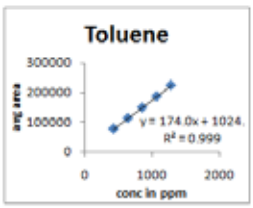

Fig 5f: Calibration plot of Toluene.

Figure 5: Calibration plots of residual solvents

Table 2: System suitability data

Solvent Name	Retention Time (n=6)	Avg. Area (n=6)	Resolution	Tailing Factor (n=6)	Theoretical Plates (n=6)	SD (n=6)	%RSD
Methanol	4.897	252505	0	1.209	29300.249	21655	8.58
Acetone	7.475	800064	19.327	1.046	38146.006	52295	6.54
IPA	7.831	421641	2.245	1.048	36290.141	36653	8.69
MDC	8.436	29699	3.919	1.011	53971.394	2503	8.43
EA	10.972	646795	17.434	1.023	89695.215	45888	7.09
Toluene	16.447	192033	37.304	1.015	198228.448	17387	9.05

Table 3: Specificity data

Solvent Name	Retention Time (min)	
	Individual	Spiked
Methanol	4.925	4.968
Acetone	7.508	7.561
IPA	7.867	-
MDC	8.478	-
EA	11.020	-
Toluene	16.505	16.513

Table 4: Method precision data

Solvent Name	Avg. Area(n=6)	SD (n=6)	%RSD
Methanol	3685	327.7273	8.89
Acetone	1214.66	148.2101	12.20
Toluene	7243.5	277.4619	3.83

Table 5: Linearity data

Solvent Name	Average Area(n=3)					Correlation coefficient	Regression Equation
	Concentration						
	50%	75%	100%	125%	150%		
Methanol	97516	164291	231066	273699	316332	0.9985	y = 73.657x –2203.9
Acetone	417900	637925	857950	1028793	1199639	0.9986	y = 158.96x - 46703
IPA	221821	366309	510798	601335	691871	0.9985	y = 95.169x –8380.3
MDC	21466	31607	41748	56432	71116	0.9987	y = 75.848x –5176.2
EA	299006	463696	628386	746330	864273	0.9998	y = 113.76x - 35071
Toluene	76042	112568	149093	187023	224953	0.9999	y = 174.02x - 22343

Table 6: LOD data

Peak	Name	Level S/N	Detection Limit	Avg. Area (n=6)	SD (n=6)	%RSD
1	Methanol	1589.835287	0.001887	960	96	9.97
2	Acetone	3848.696745	0.000779	652	31	4.79
3	IPA	1945.177844	0.001542	837	60	7.21
4	MDC	155.049969	0.019349	153	6	4.15
5	EA	3286.279342	0.000913	381	22	5.90
6	Toluene	983.603468	0.003050	933	50	5.34

Table 7: LOQ data

Peak#	Name	Level S/N	Quantitation Limit	Avg. Area (n=6)	SD (n=6)	%RSD
1	Methanol	1589.835287	0.006227	3109	29	0.94
2	Acetone	3848.696745	0.0025707	2369	36	1.52
3	IPA	1945.177844	0.005886	2744	51	1.83
4	MDC	155.049969	0.0638517	552	21	3.88
5	EA	3286.279342	0.0030129	1809	24	1.33
6	Toluene	983.603468	0.010065	3430	44	1.30

Table 8: Robustness data

Solvent Name	Avg. Area in mixed standard solution (n=6)			Test		
		(PPM) **(n=6)**				
	Org-Temp(35°c) & Flow	Temp 40°c	Temp30°c	Org-Temp(35°c) & Flow	Temp 40°c	Temp 30°c
Methanol	256496	249066	252115	44	43	43
Acetone	801497	774294	798975	9	7	7
IPA	427639	393828	419812	-	-	-
MDC	31559	28848	28828	-	-	-
EA	653506	642138	643765	-	-	-
Toluene	194383	183920	190588	33	25	25

Table 9: Ruggedness data

Solvent Name	Avg. Area (n=6)				**Test** (PPM) **(n=6)**			
	Ana-1	Ana-2	Day-1	Day-2	Ana-1	Ana-2	Day-1	Day-2
Methanol	3881	3894	3800	3870	46	46	44	45
Acetone	1190	1454	1376	1195	7	9	9	7
Toluene	7224	7486	7019	7223	33	34	32	33

Table 10: Accuracy data for sample solution

Solvents	Level	Avg. peak area (n=3)			%Recovery	Mean % Recovery
		Non spiked solution	Spiked solution	Standard solution		
Methanol	50%	95589	1927	97516	96.04	96.34
	100%	227213	3853	231066	96.66	
	150%	310552	5780	316332	96.34	
Acetone	50%	417209	691	417900	99.66	99.66
	100%	856569	1381	857950	99.67	
	150%	1197564	2072	1199639	99.65	
Toluene	50%	72396	3646	76042	90.41	90.29
	100%	141801	7292	149093	90.21	
	150%	214015	10938	224953	90.27	

Table 11: Batch analysis data for sample solution

Solvent Name	Avg. Area (n=2)	Avg. ppm (n=2)	Actual ppm (limit)
Methanol	3263.5	39	3000
Acetone	1229.5	8	5000
Toluene	7292.5	33	890

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